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Antioxidant activities of *Erythrina stricta* Roxb.using various *in vitro* and *ex vivo* models

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SUMMARY

Erythrina stricta, a deciduous tree widely used traditionally in indigenous system of medicine for various ailments such as rheumatism, fever, leprosy, epilepsy etc. The leaves of *Erythrina stricta* was extracted with ethanol (70%) and used for the evaluation of various *in vitro* antioxidant assays which includes H - donor activity, nitric oxide scavenging, superoxide anion scavenging, reducing ability, hydroxyl radical, hydrogen peroxide scavenging, total phenolic content, total flavonoid content, total antioxidant activity by thiocyanate and phosphomolybdenum method, metal chelating, β-carotene bleaching, total peroxy radical assays. The pro-oxidant activity was measured using bleomycin-dependent DNA damage. *Ex vivo* models like lipid peroxidation and erythrocyte haemolysis were also used to study the antioxidant property of the extract. The various antioxidant activities were compared with suitable standard antioxidants such as ascorbic acid, butylated hydroxyl toluene, α-tocopherol, curcumin, quercetin and Trolox. The generation of free radicals viz. $O_2^{\cdot-}$, $OH^{\cdot}$, H_2O_2, $NO^{\cdot}$ and peroxyl radicals were effectively scavenged by the ethanolic extract of *Erythrina stricta*. In all the methods, the extract offered strong antioxidant activity in a concentration dependent manner. The total phenolic content, flavonoid content and total antioxidant activity in *Erythrina stricta* were determined as microgram (g) pyrocatechol, quercetin and α-tocopherol equivalent/mg respectively. The extract did not exhibit any pro-oxidant activity when compared with ascorbic acid. The results obtained in the present study clearly indicates that *Erythrina stricta* scavenges free radicals and reduces lipid peroxidation, ameliorating the damage imposed by oxidative stress in different disease conditions and serve as a potential source of natural antioxidant.

Key words: *Erythrina stricta* (Papilionaceae); Reactive oxygen species; Free radicals; Lipid peroxidation; Antioxidants

INTRODUCTION

Free radicals have an unpaired electron in the outer orbit and are usually unstable and very reactive, causing oxidation of biomolecules such as protein, amino acids, lipid and DNA leading to cell injury and death (Freidovich, 1999; Gilbert, 2000; McCord, 2000). Oxygen, a vital component found in the atmosphere is a stable triplet biradical ($^3O_2$), is required for the survival of the human being. The most important free radicals in the body are the derivatives of oxygen, which are known as

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reactive oxygen species. Oxygen is also required for the generation of reactive nitrogen species and reactive chlorine species (Nordberg and Arner, 2001). It undergoes reduction and during these process small amounts of reactive intermediates like superoxide anion (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are formed (Sies, 1993). Free radicals react with other compounds trying to capture the electrons needed to gain stability, but rendering the substrate radical nature. These highly reactive radicals can start a chain reaction initiating lipid peroxidation which results in destabilization and disintegration of the cell membranes resulting in the disruption of cells leading to ischemic-reperfusion injury, diabetes, allergy etc. (Yamauchi *et al.,* 1992; Mates *et al.,* 1998; Vendemiale *et al.,* 1999; Kaur and Kapoor, 2001). Oxidative stress, which releases oxygen radicals in the body, which has been implicated in number of diseases such as cardiovascular malfunction, cancer, atherosclerosis, cataract, gastric ulcer, rheumatism and other autoimmune diseases like aging (Ames, 1983; Steinberg, 1991; Steinmetz and Potter, 1996; Das *et al.,* 1997). Search of naturally occurring antioxidant compounds from plant sources might serve as leads for the development of novel drugs, which may reduce the risk of chronic diseases, caused by free radicals (Kim *et al.,* 2003).

Erythrina stricta Roxb. (Papilionaceae), a medium sized deciduous tree found in various parts of India, Thailand, Nepal, Vietnam and China, is used in the indigenous system of medicine for various ailments such as rheumatism, fever, leprosy, epilepsy etc. The plant contains alkaloids and non alkaloidal constituents (Nadkarni, 1954; Singh, 1981; Sivarajan, 1994). Literature survey of *Erythrina stricta* revealed that no work has been reported on the antioxidant activities of this plant. Therefore, we investigated the antioxidant activities of the leaves using various *in vitro* and *ex vivo* models.

MATERIALS AND METHODS

Plant materials

The leaves of *Erythrina stricta* were collected from Nilgiris district, Tamil Nadu, India during the month of August 2006 and were shade dried. The leaves were identified and authenticated by G.V.S Murthy, Joint Director, Botanical Survey of India, Tamilnadu Agriculture University Campus, Coimbatore. (Ref. No. BSI/SC/5/23/06-07/Tech 641). The voucher specimen is available in the herbarium file of our department.

Preparation of extract

The dried leaves were pulverized into fine powder using a grinder and sieved through No. 22 mesh sieve and stored in an air tight container. About 750 ml of 70% ethanol was added to 75 g of powder and kept on a mechanical shaker for 72 h, the content was filtered and concentrated under reduced pressure, under controlled temperature of 40 ºC, to yield a dark gummy residue. The concentrated extract was stored dry at 4 ºC in amber coloured jars with Teflon lined caps. The percentage yield of the *Erythrina stricta* ethanolic extract (ESEE) was found to be 7.2% w/v.

Drugs and chemicals

2,2 Diphenyl-1-picryl hydrazyl hydrate (DPPH) was procured from Himedia, Mumbai, xanthine oxidase, quercetin, hypoxanthine, pyrocatechol were purchased from SRL, Mumbai, Folin Ciocalteu reagent from SD Fine Ltd, Mumbai, calf thymus DNA from Genei chemicals, Bangalore, ferrozine, (2'-2' azobis (2-amidinopropane) dihydrochloride) and Trolox obtained from Sigma Aldrich, USA, and 2,7-dichloro flurescein diacetate from Fluka, Germany. All other chemicals used in the study were of analytical grade procured from local suppliers.

Experimental animals

Wistar albino rats of either sex (150 - 200 g) were

used for the *ex vivo* study. They were housed in standard polypropylene cages and kept under controlled room temperature (24 ± 20 ºC, relative humidity 45 - 55%) in a 12 h light-dark cycle. The rats were given a standard laboratory diet and water *ad libitum.* The study was conduted after ethical clearance from the institutional animal ethics committee bearing the reference number 817/04/ac/CPCSEA.

Phytochemical screening

Preliminary phytochemical screening of the powdered leaves was performed for the presence of alkaloids, phenolics, flavonoids, saponins, carotenoids, carbohydrates and glycosides (Khandelwal, 2004).

***In vitro* antioxidant activity**

DPPH radical scavenging assay

The hydrogen donating ability of ESEE was examined in the presence of DPPH stable radical (Mensor *et al.,* 2001). One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentrations and allowed to react at room temperature. After 30 min the absorbance values were measured at 517 nm. Ethanol (1.0 ml) plus plant extract solution (2.5 ml) was used as a blank, DPPH solution (1.0 ml, 0.3 mM) plus ethanol (2.5 ml) served as negative control. The positive controls were those using the standard (Ascorbic acid) solutions.

Nitric oxide radical scavenging assay

Various concentrations of the ESEE and sodium nitroprusside (10 mM) in phosphate buffer saline (0.025 M, pH 7.4) in a final volume of 3 ml was incubated at 25 ºC for 150 min. Control experiments without the test compounds but with equivalent amount of buffer were prepared in the same manner as done for the test. There after, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess' reagent (1% sulphanilamide, 2% O-Phosphoric acid and 0.1% naphthyethylene diamine dihydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthyethylene diamine dihydrochloride was read at 546 nm. The percentage inhibition was calculated. The experiment was done in triplicate using curcumin (50 - 800 µg/ml) as positive control (Sreejayan and Rao, 1997).

Deoxyribose degradation assay

The decomposing effect of ESEE on hydroxyl radicals was determined by the assay of malondialdehyde chromogen formation due to 2-deoxy 2-ribose degradation (Halliwell *et al.*, 1987). The assay mixture contained in a final volume of 1 ml: 100 µl of 28 mM 2-deoxy 2-ribose dissolved in phosphate buffer, pH 7.4, 500 µl of the plant extract of various concentrations in buffer, 200 µl of 200 mM ferric chloride (1: 1 v/v) and 1.04 mM EDTA and 100 µl of 1.0 mM hydrogen peroxide and 100 µl of 1.0 µM ascorbic acid. After incubation of the test sample at 37 ºC for 1 h the extent of free radical damage imposed on the substrate deoxyribose was measured using thiobarbituric acid (TBA) test. Percentage inhibition of deoxyribose degradation was calculated. Quercetin was used as standard.

NBT reduction assay

A reaction mixture with a final volume of 3 ml per tube was prepared with 1.4 ml of 50 mM KH_2PO_4-KOH, pH 7.4 containing 1 mM EDTA, 0.5 ml of 100 µm hypoxanthine, 0.5 ml of 100 µM NBT (Guzman *et al.*, 2001). The reaction was started by adding 0.066 units per tube of xanthine oxidase freshly diluted in 100 µl of phosphate buffer and 0.5 ml of test extract in saline. The xanthine oxidase was added last. The subsequent rate of NBT reduction was determined on the basis of spectrophotometric determinations of absorbance at 560 nm. Ascorbic acid was used as standard. The results are expressed as the percentage inhibition of NBT reduction rate with respect to the reaction mixture without test compound (saline only).

Reducing power ability

Reducing power ability was measured by mixing 1.0 ml extract of various concentration prepared with distilled water to 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50 ºC for 30 min. After that 2.5 ml of trichloroacetic acid (10%) were added to the mixture and centrifuged for 10 min at 3,000 g, 2.5 ml from the upper part were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1%, ferric chloride. The absorbance was measured at 700 nm using UV-spectrophotometer. The reference solution was prepared as above, but contained water instead of the samples. Increased absorbance of the reaction mixture indicates increased reducing power. All experiments were done in triplicate using butylated hydroxyltoluene (BHT) as positive control (Oyaizu, 1986).

Estimation of total phenolic component

Total soluble phenolics of the extract was determined with Folin-Ciocalteu reagent using pyrocatechol as a standard following the method of Slinkard and Singleton (1977). One millilitre (1.0 ml) of extract solution in a test tube was added to 0.2 ml of Folin Ciocalteu reagent (1: 2 in distilled water) and after 20 min, 2.0 ml of purified water and 1.0 ml of sodium carbonate (15%) was added. Allowed to react for 30 min and then absorbance was measured at 765 nm. The concentration of total phenolic component in the extract was determined as microgram of pyrocatechol equivalent.

Total flavonoid content

Total soluble flavonoid of the extract was determined with aluminium nitrate using quercetin as a standard (Hsu, 2006). Plant extract (1000 μg) was added to 1ml of 80% ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10% aluminium nitrate, 0.1 ml of 1 M potassium acetate and 4.3 ml of 80% ethanol. The absorbance of the supernatant was measured at 415 nm after 40 min at room temperature. Total flavonoid concentration was calculated using quercetin as standard.

Phosphomolybdate method

The total antioxidant capacity of the extract was determined with phosphomolybdenum using α-tocopherol as the standard. An aliquot of 0.1 ml of ESEE (100 μg) solution was combined with 1.0 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 ºC for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in UV spectrophotometer.

The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as equivalents of α-tocopherol (Jayaprakasha *et al.,* 2002).

Bleomycin-dependent DNA damage

The reaction mixture contained 0.5 ml calf thymus DNA (10 μg/ml), 50 μg of 1.0 ml bleomycin sulfate, 1.0 ml of 5 mM magnesium chloride, 1.0 ml of 50 μM ferric chloride and 1.0 ml of different concentrations of ESEE. The mixture was incubated at 37 ºC for 1 h. The reaction was terminated by addition of 0.05 ml EDTA (0.1 M). The colour was developed by adding 0.5 ml TBA (1% w/v) and 0.5 ml hydrochloric acid (25% v/v) followed by heating at 37 ºC for 15 min. After centrifugation the extent of DNA damage was measured in a UV-spectrophotometer at 532 nm employing ascorbic acid as positive control. Each determination was done in triplicate (Ng *et al.,* 2003).

Thiocyanate method

The peroxy radical was determined by thiocyanate method using α-tocopherol as standard (Gutierrez *et al.,* 2006). Increasing concentration of the samples (25 - 400 μg/ml) in 0.5 ml of distilled water was

mixed with 2.5 ml of linoleic acid emulsion (0.02 M, in 0.04 M pH 7.0 phosphate buffer) and 2 ml phosphate buffer (0.04 M, pH 7) in a test tube and incubated in darkness at 37 ºC. At intervals during incubation, the amount of peroxide formed was determined by reading absorbance of red color developed at 500 nm by the addition of 0.1 ml of 30% ammonium thiocyanate solution and 0.1 ml of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture.

Hydrogen peroxide scavenging activity

Hydrogen peroxide solution (2 mM) was prepared with standard phosphate buffer (pH, 7.4). Extract samples (25 - 400 μg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined spectrophotometrically after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of both plant extract and standard compound (α-tocopherol) was determined (Oktay *et al.,* 2003).

Metal chelating complex

The ferrous level was monitored by measuring the formation of the ferrous ion-ferrozine complex. The reaction mixture containing different concentrations of ESEE (1.0 ml) were added to 2 mM ferrous chloride (0.1 ml) and 5 mM ferrozine (0.2 ml) to initiate the reaction and the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The positive control were those using ascorbic acid and all tests and analysis were run in triplicate. The percentage chelating effect of Ferrozine-Fe^{2+} complex formation was calculated (Huang and Kuo, 2000).

β-carotene linoleic acid assay

A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml chloroform and 1.0 ml of this solution was then pipetted into a flask containing 20 mg of linoleic acid and 200 mg of Tween 40 emulsifier. Chloroform was completely evaporated using a vaccum evaporator. Aliquots of 5.0 ml of this emulsion were transferred into a series of tubes containing various concentration of ESEE (25 - 400 μg/ml) or α-tocopherol for comparison. Optical density (OD) at 470 nm was taken for ESEE and standard immediately (t = 0) and at the end of 90 min (t = 90). The tubes were incubated at 50 ºC in a water bath during the test. Measurement of OD was continued until the colour of β-carotene disappeared in the control (Jayaprakasha *et al.,* 2002).

Total peroxy radical trapping potential (TRAP)

A water soluble azo initiator 2,2' azo bis (2-amidino propane) dihydrochloride (AAPH) produced the peroxyl radicals while a spectrophotometric analysis of 2,7-dichlorofluresecin-diacetate (DCF) monitored the scavenging activity of the plant extracts. A 350 μl of 1 mM stock of DCF in ethanol was mixed with 1.75 ml of 0.01 N sodium hydroxide and allowed to stand for 20 min before the addition of 17.9 ml of 25 mM sodium phosphate buffer (pH 7.2). The reaction mixture contained 0.5 ml of various concentration of ESEE in ethanol, 150 μl of activated DCF solution and 25 μl of AAPH (56 mM). The reaction was initiated with the addition of the AAPH. Absorbance was read at 490 nm. Trolox (6-hydroxy 2,5,7-8 tetra methyl chroman 2 carboxylic acid) was used as standard and all the determination was done in triplicate (McCune and John, 2002).

***Ex vivo* studies**

Assay of lipid peroxidation method

Lipid peroxidation induced by Fe^{2+} -ascorbate system in rat liver homogenate was estimated by TBA reaction method (Ohkawa *et al.,* 1979). The reaction mixture consisted of rat liver homogenate 0.1 ml (25% w/v) in Tris-HCL buffer (20 mM, pH 7.0), potassium chloride (30 mM), ferrous ammonium sulphate (0.16 mM), ascorbate (0.06 mM), and various concentrations of the ESEE in a final volume of 0.5 ml.

The reaction mixture was incubated for 1 h at 37 ºC. After the incubation time, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS) (8.1%), 1.5 ml TBA (0.8%), and 1.5 ml glacial acetic acid (20%, pH 3.5). The total volume was made upto 4 ml by distilled water and then kept in a water bath at 95 - 100 ºC for 1 h. After cooling, 1.0 ml of distilled water and 0.5 ml of n-butanol and pyridine mixture (15: 1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4,000 g for 10 min. The organic layer was removed and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by comparing the OD of the treatments with that of control. Ascorbic acid was used as standard.

Assay of erythrocyte hemolysis

The blood was obtained from human volunteers and collected in heparinized tubes. Erythrocytes were separated from plasma and the buffy coat was washed three times with 10 volumes of 0.15 M sodium chloride. During the last wash, the erythrocytes were centrifuged at 3,000 g for 10 min to obtain a constantly packed cell preparation. Erythrocyte hemolysis was mediated by peroxyl radicals in this assay system. A 0.2 ml of 10% suspension of erythrocytes in phosphate buffered saline pH 7.4 (PBS) was added to the similar volume of 200 mM 2, 2' azobis (2 amidinopropane) dihydrochloride (AAPH) solution (in PBS) containing samples to be tested at different concentrations. The reaction mixture was shaken gently while being incubated at 37 ºC for 2 h. The reaction mixture was then removed, diluted with eight volumes of the PBS and centrifuged at 2,000 g for 10 min. The absorbance of the supernatant was read at 540 nm (A). Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete hemolysis, and the absorbance (B) of the supernatant obtained after centrifugation was measured at 540 nm. The data were expressed as mean ± S.E.M. L-ascorbic acid was used as a positive control (Ng *et al.,* 2000).

Calculation of 50% inhibitory concentration (IC_{50})

The concentration (mg/ml) of the extract required to scavenge 50% of the radicals was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I%) was calculated using the formula,

$$I\% = \frac{(Ac - As)}{Ac} \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

RESULTS

Phytochemical screening

Phytochemical screening of the plant extract revealed the presence of alkaloids, flavonoids and saponins.

Hydrogen donating assay

The radical scavenging activity of crude ESEE was determined from the reduction in the optical absorbance at 517 nm due to scavenging of stable DPPH free radical. Positive DPPH test suggests that the extract is a potential free radical scavenger. ESEE showed strong activity compared with the standard, ascorbic acid. Table 1 shows the IC_{50} values of the sample and the standard.

NO assay

Incubation of solutions of sodium nitroprusside in phosphate buffered saline at 25 ºC for 150 min resulted in the generation of NO. The ESEE effectively reduced the generation of $NO^{\cdot}$. This showed marked $NO^{\cdot}$ scavenging activity of the extract. The IC_{50} was found to be 0.157 mg/ml for ESEE and for standard, curcumin, it was found to be 0.076 mg/ml (Table 1).

Deoxyribose degradation

The scavenging effect of ESEE on hydroxyl ($OH^{\cdot}$) was quantified by measuring the effect on the 2-deoxy-ribose degradation produced by reacting

Table 1. Antioxidant activity of *Erythrina stricta* by different models

	In vitro methods - IC_{50} (mg/ml)								*Ex-vivo* methods IC_{50} (mg/ml)	
DRUG	DPPH	$NO^{\cdot}$	$OH^{\cdot}$	$O_2^{\cdot -}$	H_2O_2	Thiocyanate method	Metal chelating	TRAP assay	Lipid peroxidation	Erythrocyte hemolysis
ESEE	0.081 ± 1.66	0.157 ± 19.6	0.32 ± 12.0	0.085 ± 2.88	0.068 ± 4.41	0.176 ± 6.66	0.052 ± 2.45	0.146 ± 7.26	0.263 ± 3.33	0.173 ± 7.26
Ascorbic acid	0.003 ± 0.06			0.072 ± 12.6			0.044 ± 26.8		0.081 ± 4.41	0.110 ± 0.66
Quercetin			0.112 ± 11.2							
Curcumin		0.076 ± 9.33								
α-tocopherol					0.066 ± 1.66	0.09 ± 3.33				
Trolox								0.099 ± 2.33		

Fe^{3+} with ascorbate, in the presence of EDTA. The IC_{50} value of ESEE was 0.32 mg/ml and that of standard, quercetin was 0.112 mg/ml (Table 1).

Superoxide radical scavenging activity

ESEE suppressed the superoxide anion radicals generated from hypoxanthine/xanthine oxidase system. Inhibition of NBT reduction by superoxide in the presence of the test preparation increased with raise of their concentrations. All measurements were compared with control experiment. The results shows that ESEE had antioxidative activity similar to the positve control, BHT (Table 1).

Reducing power

The reductive ability of the extract served as a significant indicator of its potential antioxidant activity. ESEE and standard (BHT) were used at dose range of 50 - 800 μg/ml. The reducing power of ESEE increased concentration dependently. All concentrations of the extract offered higher activities than control (Table 2).

Total phenolic, flavonoid contents and total antioxidant capacity

The content of total phenolics in ESEE was determined using Folin-Ciocalteu assay, calculated from regression equation of calibration curve of pyrocatechol. Phenolic content of ESEE was found to be 110 g pyrocatechol equivalent/mg. The total flavonoid content of ESEE was found to be 528 g quercetin equivalent/mg. The total antioxidant capacity of ESEE was found to be 8 μg α-tocopherol equivalent/mg.

Bleomycin-dependent DNA damage

The pro-oxidant activity of ESEE and the standard, ascorbic acid are represented in Table 4. ESEE and

Table 2. Reducing power ability

Drug	Absorbance at 700 nm				
	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml	800 μg/ml
ESEE	0.061 ± 0.003	0.097 ± 0.005	0.168 ± 0.006	0.225 ± 0.006	0.333 ± 0.001
BHT	0.092 ± 0.002	0.214 ± 0.004	0.314 ± 0.004	0.640 ± 0.001	1.092 ± 0.008

Table 3. β-Carotene bleaching inhibitory activity

Fractions	Time of incubation (min)	Absorbance at 470 nm					IC_{50} (mg/ml)
		25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml	
ESEE	0	0.091	0.127	0.179	0.278	0.718	0.098 ± 0.05
	90	0.062	0.066	0.138	0.228	0.622	
α-Tocopherol	0	0.098	0.126	0.189	0.285	0.725	0.100 ± 0.01
	90	0.058	0.082	0.138	0.230	0.664	

Table 4. Pro-oxidant activity of different fractions of *Erythrina stricta*

Drugs	Absorbance at 532 nm				
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml
ESEE	0.244 ± 0.004	0.235 ± 0.005	0.178 ± 0.014	0.108 ± 0.018	0.038 ± 0.01
Ascorbic acid	0.805 ± .016	0.655 ± 0.009	0.425 ± 0.008	0.150 ± 0.005	0.035 ± 0.001

ascorbic acid were tested at concentrations ranging from 25 - 400 g/ml. ESEE decreased the absorbance and the bleomycin-Fe^{3+} is not converted into bleomycin-Fe^{2+}, thereby preventing the DNA degradation suggesting that ESEE is devoid of pro-oxidant activity.

Thiocyanate method

The total antioxidant activity of the ESEE was determined and compared with that of standard, α-tocopherol by thiocyanate method. ESEE exhibited effective antioxidant activity at all doses and increased concentration dependently and IC_{50} value was found to be 0.176 mg/ml. The standard, α-tocopherol showed the IC_{50} value of 0.933 mg/ml (Table 1).

Hydrogen peroxide scavenging assay

ESEE was capable of scavenging H_2O_2 in an amount dependent manner. The scavenging ability of the extract and standard, α-tocopherol are shown in Table 1. H_2O_2 scavenging activity of ESEE was closer to that of α-tocopherol at doses of 100, 200 and 400 μg/ml.

Ferrous chelating ability

The ability to chelate ferrous ions also increased with an increase in ESEE concentration, which indicates that ESEE chelates the iron ions. The metal chelating effect of ESEE was lower than the standard, ascorbic acid. The values shown in Table 1 demonstrate the action of ESEE, as peroxidation protector.

β-Carotene bleaching method

The antioxidant activity of ESEE and the standard drug α-tocopherol were evaluated by β-carotene bleaching method and the results are presented in Table 3. ESEE and α-tocopherol were used in the concentration between 25 - 400 mg/ml and an increase in concentration of the extract and standard decreased the absorbance and this was due to the inhibition of bleaching of the colour of β-carotene. The 50% inhibition value for ESEE was 0.098 mg/ml and for α-tocopherol was 0.1 mg/ml. ESEE exhibited equivalent β-carotene bleaching activity when compared with α-tocopherol.

Total radical antioxidant potential (TRAP)

The peroxyl radical scavenging activity was determined for ESEE and the results were compared with Trolox (Table 1). Addition of increasing concentration of ESEE to solution containing AAPH-derived peroxyl radical decreased the luminescence produced by DCF and the absorbance decreased in a linear fashion. ESEE and Trolox exhibited IC_{50} values of 0.146 and 0.099 mg/ml respectively.

Lipid peroxidation

ESEE was effective in inhibiting the lipid peroxidation induced by Fe^{2+}- ascorbate system in rat liver homogenate. The MDA generated as a result of lipid peroxidation reacts with thiobarbituric acid and was found to be inhibited in the presence of the extract. The IC_{50} value was found to be 0.263 mg/ml for ESEE while for standard ascorbic acid the IC_{50} was found to be 0.081 mg/ml (Table 1).

Erythrocyte haemolysis

The peroxyl radical generated by AAPH on addition to erythrocyte suspension and its subsequent scavenging action produced by graded concentrations of ESEE and standard, ascorbic acid are given in Table 1. An increase in inhibition was noticed at all concentrations of the extract and ascorbic acid.

DISCUSSION

Antioxidants exert their mode of action by suppressing the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements (Cioffi *et al.,* 2002). DPPH assay is a widely used method to evaluate the radical scavenging effect of natural antioxidants (Brand-Williams *et al.,* 1995). DPPH is a stable free radical, which produces a deep violet colour solution in ethanol at room temperature. Antioxidants reduce DPPH to diphenyl 2-picryl hydrazine, giving rise to yellow colored at 517 nm (Oyaizu, 1986; Illavarasan *et al.,* 2005). The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups. We can infer that, the very good activity of the extract may be probably due to the presence of substances with an available hydroxyl group.

NO has an unpaired electron and acts as a free radical which is implicated in inflammation, cancer and other pathological conditions (Toda *et al.,* 1988; Moncada *et al.,* 1991). NO generated from sodium nitroprusside in aqueous solution at physiological pH, spontaneously interacts with oxygen to produce nitrite ions, which is estimated by the use of Griess reagent. Scavengers of NO compete with oxygen leading to the reduced production of NO (Marcocci *et al.,* 1994). Our finding suggests that the phenolic components present in the extract might be responsible for NO scavenging effect.

Hydroxyl radicals are highly reactive oxygen centered radicals causing lipid oxidation and enormous biological damage (Koppenol, 1993). Hydroxyl radicals produced in free solution were detected by their ability to degrade 2-deoxy-2-ribose into fragments, which forms a pink chromogen (Fenton reaction) upon heating with TBA at low pH. When ESEE and reference compound quercetin were added to the reaction mixture, they removed hydroxyl radicals from the sugar and prevented their degradation.

Superoxide anions serve as precursors of singlet oxygen and hydroxyl radicals (Kumar *et al.,* 2005). The superoxide anions generated by hypoxanthine/ xanthine oxidase system reduce nitroblue tetrazolium to form a chromophore (diformazan) that absorbs at 560 nm (Kirby and Schmidt, 1997). The exract decreased the mean rate of absorption by inhibiting NBT reduction by the superoxide anion radicals.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing ability is generally associated with the presence of reductones, which breaks the free radical chain by donating a hydrogen atom (Oyaizu, 1986; Gordon, 1990). The extract had reductive ability which increased with increasing concentrations of the extract.

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen (Javanmardi *et al.,* 2003). Flavonoids are wide spread in all natural compounds and possess a broad spectrum of biological activities (Agrawal, 1989). The chemical composition of *Erythrina stricta* indicates the presence of phenolic compounds including tannins and flavonoids, which are known to

possess antioxidant activities (Motalleb *et al.,* 2005). The high phenolic and flavonoid content in the ethanolic extract of *Erythrina stricta* may be responsible for its free radical scavenging activity.

The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as -tocopherol equivalent (Prieto *et al.,* 1999). The extract reduced molybdenum VI to a green coloured phosphomolybdenum V complex, which proves its total antioxidant capacity.

Bleomycin binds iron ions and the bleomycin-Fe^{3+} complex degrades DNA in the presence of O_2 and ascorbic acid, which reduces Fe^{3+} to Fe^{2+}at high concentrations (Aruoma, 2003). The bleomycin-Fe^{3+} complex by itself is inactive in inducing damage to DNA. Oxygen and reducing agent or H_2O_2 are required for the damage of DNA to occur. The extract decreased the absorbance and bleomycin-Fe^{3+} is not converted into bleomycin-Fe^{2+} thereby preventing the DNA degradation. The results confirm that ESEE is devoid of pro-oxidant activity.

The thiocyanate method measures the amount of peroxides produced at the initial stage of lipid peroxidation which is depicted by a decrease in absorbance indicating increased level of antioxidant activity (Gutierrez *et al.,* 2006). The good antioxidant activity exhibited by our plant extract might be attributed to the presence of flavonoid like phytochemicals.

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it can give rise to hydroxyl radicals. Thus, the removal of H_2O_2 as well as $O_2^{\cdot-}$ is very important for antioxidant defense in cell or food systems (Gulcin *et al.,* 2004). Our extract scavenged H_2O_2, which may be attributed to the presence of phenolics, which could donate electrons thereby neutralising it into water.

Metal chelating ability is based on the chelating effect of Fe^{2+} ions by ferrozine. The formation of complex is disrupted by other chelating agent, which results in the reduction of the formation of red colour in the complex (Dinnis *et al.,* 1994). In this method both the extract and standard compound (ascorbic acid) interfered with the formation of ferrous complex with the reagent ferrozine, suggesting that it has chelating effect and captures the ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex decreased linearly in a dose-dependent manner. It is reported that certain phenolic compounds have properly oriented functional groups, which can chelate metal ions (Thompson *et al.,* 1976). The ion chelating activity of the extract may be attributed due to the presence of endogenous chelating agents, mainly phenolics.

The decoloration of β-carotene is a commonly employed method to measure the antioxidant activity of plant extracts, because β-carotene is extremely sensitive to free radical mediated oxidation of linoleic acid. As β-carotene molecules lose their double bonds by oxidation, the compound loses its yellow colour (Elzaawely *et al.,* 2005). The ethanolic extract of *Erythrina stricta* inhibited β-carotene oxidation suggesting that the antioxidant activity could be related to the high levels of phenolic compounds.

TRAP assay is based upon the potential of antioxidants in extract to scavenge peroxyl radicals generated by thermal decomposition of a water-soluble azo initiator AAPH (Khanam *et al.,* 2004). *Erythrina stricta* ethanolic extract decreased the absorbance upon increasing concentrations of the sample, which is similar to that of the standard, Trolox.

Oxidative stress can lead to peroxidation of cellular lipids and can be measured by the determining the levels of thiobarbituric acid reactive substances (Yokozawa *et al.,* 2000; Zin *et al.,* 2002). Quantification of MDA, one of the products of lipid peroxidation, with TBA at low pH and high temperature (100 ºC) resulted in the formation of a red complex, which is measured at 532 nm. Our extract ESEE inhibited the rate of lipid peroxidation by a reduction in the red colour complex formed reflecting its anti-lipid peroxidative

potential.

The azo compound generates free radicals by its unimolecular thermal decomposition. The rate of generation of peroxyl radicals can be easily controlled and measured by adjusting the concentration of AAPH. Therefore, the hemolysis induced by AAPH clearly demonstrates the oxidative erythrocyte membrane damage by peroxyl radicals (Haraguchi *et al.,* 2002). The ethanolic extract of *Erythrina stricta* inhibited the erythrocyte haemolysis induced by AAPH in a concentration dependent manner.

Based on the various *in vitro* and *ex vivo* assays, it can be concluded that the ethanolic extract of *Erythrina stricta* possess strong antioxidant activity, evidenced by the free radical scavenging property, which may be due to the presence of phenolic components in the extract. Further isolation of bioactive constituents in the extract would certainly help to ascertain its potency which could be further exploited by *in vivo* study systems to increase the overall antioxidant activity by protecting against various ailments that are induced by oxidative stress.

REFERENCES

Agrawal PK. (1989) Carbon-13 NMR of flavonoids. New York: Elsevier.

Ames BN. (1983) Dietary carcinogens and anticarcinogens oxygen radicals and degenerative diseases. *Science* **221**, 1256-1262.

Aruoma OI. (2003) Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutat. Res.* **523-524**, 9-20.

Brand Williams W, Cuvelier ME, Berset C. (1995) Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* **28**, 25-30.

Cioffi G, D'Auria M, Braca A, Mendez J, Castillo A, Morelli I, De Simone F, De Tommasi N. (2002) Antioxidnt and free radical scavenging activity of constituents of the leaves of *Tachigalia paniculata*. *J. Nat. Prod.* **65**, 1526-1529.

Das D, Bandyopadhyay D, Bhattacharjee M, Banerjee RK. (1997) Hydroxyl radicals is the major causative factor in stress induced gastric ulceration. *Free Radic. Biol. Med.* **23**, 8-18.

Dinis TCP, Madeira, VMC, Almeida LM. (1994) Action of phenolic derivatives (acetoaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* **315**, 161-169.

Elzaawely AA, Xuan TD, Tawata S. (2005) Antioxidant and antibacterial activities of *Rumex japonicus* HOUTT. Aerial parts. *Biol. Pharm. Bull.* **28**, 2225-2230.

Freidovich I. (1999) Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann. NY. Acad. Sci.* **893**, 13.

Gilbert DL. (2000) Fifty years of radical ideas. *Ann. NY. Acad. Sci.* **899**, 1.

Gordon MH. (1990) The mechanism of antioxidant action in vitro. In: Food antioxidants, edited by Hudson BJF, p. 1-18. Elsevier Applied Science, London.

Gulcin I, Kufrevioglu OI, Oktay M, Buyukokuroglu ME. (2004) Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.). *J. Ethnopharmacol.* **90**, 205-215.

Gutierrez RMP, Luna HH, Garrido SH. (2006) Antioxidant activity of *Tagetes erecta* essential oil. *J. Chilean Chem. Soc.* **51**, 883-886.

Guzman S, Gato A, Calleja JM. (2001) Antiinflammatory, analgesic and free radical scavenging activities of the marine microalgae *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytother. Res.* **15**, 224-230.

Halliwell B, Gutteridge JMC, Arouma OI. (1987) The deoxyribose method: a simple 'test tube' assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* **165**, 215-219.

Haraguchi H, Inoue J, Tamura Y, Mizutani K. (2002) Antioxidative components of *Psoralea corylifolia*. *Phytother. Res.* **16**, 539-544.

Hsu CY. (2006) Antioxidant activity of extract from *Polygonum aviculare* L. *Biol. Res.* **39**, 281-288.

Huang SC, Kuo JC. (2000) Concentrations and antioxidative activity of anserine and carnosine in poultry meat extracts treated with demineralization and papain. *Proc. Natl. Sci. Counc. Repub. China B* **24**, 193-201.

Illavarasan R, Mallika M, Venkataraman S. (2005) Anti-inflammatory and antioxidant activities of Cassia fistula Linn bark extracts. *Afr. J. Trad. Comp. Alt. Med.* **2**, 70-85.

Javanmardi J, Stushnoff C, Locke E, Vivanco JM. (2003) Antioxidant activity and total phenolic content of Iranian *Ocimum* accessions. *Food Chem.* **83**, 547-550.

Jayaprakasha GK, Jena BS, Negi PS, Sakariah KK. (2002) Evaluation of antioxidant activities and antimutagenicity of turmeric oil: a byproduct from curcumin production. *Verlag der Zeitschrift fur Naturforschung* (*Z. Naturforsch., C, Biosci.*) **57c**, 828-835.

Kaur C, Kapoor HC. (2001) Antioxidants in fruits and vegetables- the millennium's, health. *Int. J. Food Sci. Technol.* **36**, 703-725.

Khanam S, Shivprasad HN, Devi K. (2004) *In vitro* antioxidant screening models: a review. *Indian J. Pharm. Edu.* **38**, 180-183.

Khandelwal KR. (2004) Practical pharmacognosy, 11th ed., pp. 149-156. Nirali Prakashan, Pune.

Kim KS, Lee S, Lee YS, Jung SH, Park Y, Shin KH, Kim BK. (2003) Antioxidant activities of the extracts from the herbs of *Artemisia apiacea*. *J. Ethnopharmacol.* **85**, 69-72.

Kirby AJ, Schmidt RJ. (1997) The antioxidant activity of Chinese herbs for eczema and of placebo herbs - I. *J. Ethnopharmacol.* **56**, 103-108.

Koppenol WH. (1993) The centennial of the Fenton reaction. *Free Radic. Biol. Med.* **15**, 645-651.

Kumar RS, Sivakumar T, Sunderam RS, Gupta M, Mazumdar UK, Gomathi P, Rajeshwar Y, Saravanan S, Kumar MS, Murugesh K, Kumar KA. (2005) Antioxidant and antimicrobial activities of *Bauhinia racemosa* L. stem bark. *Braz. J. Med. Biol. Res.* **38**, 1015-1024.

Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L. (1994) The nitric oxide scavenging property of *Ginkgo biloba* extract Egb 761. *Biochem. Biophys. Res. Commun.* **201**, 748-755.

Mates JM, Perez-Gomez C, Castro IN. (1998) Antioxidant enzymes and human diseases. *Clin. Chem.* **32**, 595-603.

McCard JM. (2000) The evolution of free radicals and oxidative stress. *Am. J. Med.* **108**, 652.

McCune LM, Johns T. (2002) Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the north American boreal forest. *J. Ethnopharmacol.* **82**, 197-205.

Mensor LL, Menezes FS, Leitao GG, Reis AS, dos Santos TC, Coube CS, Leitão SG. (2001) Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytother. Res.* **15**, 127-130.

Moncada A, Palmer RMJ, Higgs EA. (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **43**, 109-142.

Motalleb G, Hanachi P, Kwa SH, Fauziah O, Asmah R. (2005) Evaluation of phenolic content and total antioxidant activity in *Berberis vulgaris* fruit extract. *J. Biol. Sci.* **5**, 648-653.

Nadkarni KM. (1954) The Indian materia medica with ayurvedic unani and home remedies, 3rd ed., pp. 509-510. Popular Prakashan, Bombay.

Ng TB, Liu F, Lu Y, Cheng CHK, Wang Z. (2003) Antioxidant activity of compounds from the medicinal herb *Aster tataricus*. *Comp. Biochem. Physiol. C* **136**, 109-115.

Ng TB, Liu F, Wang ZT. (2000) Antioxidative activity of natural products from plants. *Life Sci.* **66**, 709-723.

Nordberg J, Arner ESJ. (2001) Reactive oxygen species, antioxidants and mammalian thioredoxin system. *Free Radic. Biol. Med.* **31**, 1287-1312.

Ohkawa H, Ohishi W, Yagi K. (1979) Assay of peroxides in animal tissue by thiobarbituric acid reaction. *Anal. Biochem.* **95**, 351-358.

Oktay M, Gulcin I, Kufrevioglu I. (2003) Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. *Lebensm. Wiss. u. Technol.* **36**, 263-271.

Oyaizu M. (1986) Studies on product of browning reaction prepared from glucose amine. *Jpn. J. Nut.* **44**, 307-315.

Prieto P, Pineda M, Aguilar M. (1999) Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal. Biochem.* **269**, 337-341.

Seis H. (1993) Strategies of antioxidant defence. *Eur. J. Biochem.* **215**, 213-219.

Singh H, Chawla AS, Kapoor VK, Kumar N. (1981) Investigation of *Erythrina* Spp. IX. chemical

constituents of *Erythrina stricta* bark. *J. Nat. Prod.* **44**, 526-530.

Sivarajan VV, Balachandran I. (1994) Ayurvedic drugs and their plant sources, pp. 350-351. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi.

Slinkard K, Singleton VL. (1977) Total phenol analysis: automation and comparison with manual methods. *Am. J. Enol. Vitic.* **28**, 49-55.

Sreejayan N, Rao MNA. (1997) Nitric oxide scavenging by curcuminoids. *J. Pharm. Pharmacol.* **49**, 105-107.

Steinberg D. (1991) Antioxidants and atherosclerosis-A current assessment. *Circulation* **84**, 1420-1425.

Steinmetz KA, Potter JD. (1996) Vegetable, fruit and cancer epidemiology. *Cancer Causes Control* **2**, 325-351.

Thompson M, Williams CR, Elliot GEP. (1976) Stability of flavonoid complexes of copper(II) and flavonoid antioxidant activity. *Anal. Chim. Acta* **85**, 375-381.

Toda S, Ohnisho M, Kimura M, Nakashima Y. (1988) Action of curcuminoids on the hemolysis and lipid peroxidation of mouse erythrocytes induced by hydrogen peroxide. *J. Ethnopharmacol.* **23**, 105-106.

Vendemiale G, Grattagliano I, Altomare E. (1999) An update on the role of free radicals and antioxidant defense in human disease. *Int. J. Clin. Lab. Res.* **29**, 49-55.

Yamauchi R, Kato K, Oida S, Kanaeda J, Ueno Y. (1992) Benzyl caffeate, an antioxidative compound isolated from propolis. *Biosci. Biotechnol. Biochem.* **56**, 1321-1322.

Yokozawa T, Cho EJ, Hara Y, Kitani K. (2000) Antioxidative activity of green tea treated with radical initiator 2,2'- azobis(2-amidinopropane) dihydrochloride. *J. Agric. Food Chem.* **48**, 5068-5073.

Zin M, Abdul-Hamed A, Ojman A. (2002) Antioxidant activity of extracts from mengkudu (*Morinda citrifolia* L.) root, fruit and leaf. *Food Chem.* **48,** 5068-5073.